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A. INTRODUCTION

We have made very significant progress since the last report (two years) towards achieving the objectives of this Center as set out in the aims of the original proposal. In response to the need to develop targeted approaches for the prevention and treatment of ER– breast cancer we have organized the COE into six integrated Projects (P1-6) that are supported by two Cores (C1-2). These projects span epidemiology, pathology, functional genomics, cancer cell biology, preclinical drug development, and chemoprevention. The overall aim of the COE is to understand the causes and critical pathways underlying the development of ER– breast cancer in order to develop novel strategies for prevention and treatment. The more detailed aims of the component projects and cores are described below.

Project 1. Epidemiology and Pathologic Precursors- Hankinson, Colditz, Schnitt

We propose to draw on the existing data from the ongoing Nurses' Health Study (G. Colditz, PI. CA 87969) and sub-studies that relates histologic subtypes of benign breast disease to subsequent risk of breast cancer in the cohort (G. Colditz PI CA 46475) and that evaluate several endogenous hormones in relation to breast cancer risk (S. Hankinson PI, CA49449). We will reevaluate existing data to relate exposure information to risk of ER– tumors to determine if risk factors differ between these subtypes of breast cancer. Specifically we will test the following hypotheses

1) Markers of endogenous hormones (e.g., age at first birth, parity, age at menarche, physical activity) and endogenous hormones themselves (i.e., estradiol, testosterone) are more strongly related to ER+ tumors while family history of breast cancer and IGF-I are more strongly related to ER– tumors.

2) Use of estrogen alone increases risk of ER+ tumors, but estrogen plus progestin promotes cell division and increases risk of ER– tumors (i.e. the proportion of ER– tumors is higher among E plus P users compared to E alone).

3) ER+ benign lesions increase the risk preferentially of ER+ tumors and ER– benign lesions increase the risk preferentially of ER– tumors

Project 2. Genomic Fingerprinting-Iglehart, Richardson

This project combines investigators from the Harvard School of Public Health, the Brigham and Women's Hospital, the Dana-Farber Cancer Institute and the Whitehead Institute for Biomedical Research. ER– breast cancer comprises 30-40% of all breast cancer in U.S. women. The etiology and growth-promoting pathways in these cancers are not fully elucidated, impeding development of successful therapies. Investigators in our COE will use expression arrays and transcriptional profiling to study ER– breast cancers. This project will test the following hypotheses:

1) All, or a distinct group of, estrogen-insensitive breast cancers are distinguished from estrogen-sensitive breast cancers by the expression of clusters of genes. Natural classifications, based upon non-biased expression profiles, will define truly estrogen-sensitive from estrogen-insensitive cancers.

2) Estrogen-insensitive cancers utilize a variable, but finite number of growth-promoting pathways to escape proliferation control, differentiation and apoptotic pathways. The "fingerprints" of these pathways will classify ER– cancers into discrete groups, segregating by predominate carcinogenic pathways.

3) By comparing expression profiles to detailed studies of specific pathways, fingerprints of different biochemical pathways may be identified. These will help to rationally sub-classify ER– cancers, and suggest targets for therapy.

Project 3. Role of Growth Factor Signaling-Brugge, Roberts

We will examine the hypothesis that aberrant expression and/or responsiveness to growth factors is involved in the development and progression of ER– human breast tumors. To accomplish this, we will:

1) Examine the phenotypic changes in acinar morphogenesis induced by several growth factors that have been implicated in breast cancer.

2) Examine how expression of genes whose function or expression are known to be altered in breast cancer perturb the response of breast epithelial cells to these growth factors.

3) Establish human breast epithelial cell lines that express several levels of PI3 kinase and AKT activity. These lines will be characterized with respect to resistance to apoptotic stimuli, receptor function, and transformed phenotype. Our working hypothesis is that either too much or too little function of the PI3K pathway can promote cell death. If the lines are not transformed by expression of single activated kinases, a large array of other pathways will be tested for synergy in tumor formation. The transcriptional profiles will be obtained in collaboration with Project 2 and compared with profiles of human breast tumor samples.

4) In parallel we will engineer mice to express constitutively activated PI3K in breast epithelium and to allow tissue specific knockout of p110α and β genes in breast epithelium. This will allow us to study the role of activation of PI3K both independently and in the context of other transgenes or knock-outs. Once again transcription in tumors will be profiled for comparison to human tumors in collaboration with Project 2.

Project 4. Role of HID-5/psoriasin-Polyak, Schnitt

To better understand the role of HID-5 in the initiation, progression and clinical behavior of high-grade ER– breast carcinomas, we propose to characterize its biochemical and biological functions. Specifically we will:

1) Analyze the role of HID-5 in the regulation of mammary cell growth and survival

2) Analyze HID-5 expression in primary tumors using tissue microarrays

3) Evaluate HID-5 as a potential breast cancer biomarker

Project 5. Development of NF-κB Directed Therapy-Pardee, Ghosh

We will be to test the hypothesis that blocking the activation of the nuclear factor kappa B (NF-κB) will preferentially block the growth of the class of ER– breast cancers that over-express EGF-R family receptors.

1) Screen potential small molecule inhibitors of NF-κB in ER– breast cancer cells with activated EGF signaling pathways

2) Determine whether influencing the interactions of different components of the Ikk-complex has a significant effect on growth and proliferation of the ER– breast cancer cells

Project 6. Development of Nuclear Receptors as Targets for Prevention-Spiegelman, Brown, Garber

In this project we will test the hypothesis that nuclear receptors can be targeted for the prevention of ER– breast cancers. Specifically we will investigate the possible roles of PPARγ and various other nuclear receptors in ER– breast cancer. This will involve both the development of preclinical models for the action of these receptors in mammary epithelial cells and the translation of these findings into pilot clinical trials. The specific aims of this project are:

1) Transcription profiling of PPARγ ligand and rexinoid activation in human breast cancer cells

3) Genetic analysis of PPARγ function in the mouse breast

3) Therapeutic models of PPARγ ligands in breast cancer

4) Mine the gene expression profiling data obtained in Project 2 for nuclear receptors and their targets expressed in ER– breast cancers

Further we hypothesize that it is feasible to measure a variety of biological markers in ductal lavage specimens, and we are specifically interested in genes altered by rosiglitizone and other nuclear receptor ligands. We will:

1) Assess the feasibility of obtaining breast duct epithelial cells using ductal lavage in premenopausal women with a prior diagnosis of ER– breast cancer before and three months after the initiation of a chemoprevention agent.

2) Examine biomarkers in ductal lavage specimens before and after 3 months of a prevention agent. Specifically, pilot projects will be performed examining the impact of rosiglitizone and in the future other nuclear receptor ligands. Quantitative RT-PCR will be performed for growth regulatory genes found to be regulated by these agents in the pre-clinical models.

Core 1. Signal Transduction Rapid Assessment Core-DeCaprio, Roberts

The Luminex technology has been successfully developed for multiplexed analysis of cytokines (81). It is the goal of this proposal to identify monoclonal antibodies that can detect the activation-state of the signal transduction cascade as it specifically applies to breast cancer. The long-term goal is to develop this technology so that it can be applied to the rapid assessment of activation-state of signal transduction in primary tumor samples. Specifically this will involve:

1) Detection of phsopho-tyrosine signaling in breast cancer. Monoclonal antibodies against proteins whose activation state is heralded by the presence of phsopho-tyrosine will be evaluated. In particular, antibodies against receptor and non-receptor tyrosine kinases as well as downstream signaling molecules will be assessed on the Luminex platform with an anti-phsopho-tyrosine antibody (4G10 or related antibodies).

2) Screen pairs of antibodies for detection of activated signaling in breast cancer. Signaling molecules become activated by phosphorylation of a specific residue. Pairs of antibodies that capture a signaling molecule and recognize a specific phospho-residue will be evaluated on the Luminex system.

Core 2. Communications Core-Brown, Winer, Bernardo, Temple, Hirshfield-Bartek, Matyka

In order to facilitate the synergistic activities of the various components of the COE we will establish a Communications Core based at the Dana-Farber Cancer Institute. This Core will provide Communications, Administrative, Informatics, and Biostatistics support for the entire COE. In addition an important aspect of this core will be the involvement of Consumer Advocates. The Core will maintain the COE web site (http://research.dfci.harvard.edu/coe). In addition the Core will facilitate the development of web based tools for data analysis. The first will be the data mining tool for the "Genomic Fingerprinting" data obtained in Project 2.

B. BODY

Progress to date

Major Accomplishments

(1) The definition of the reproductive, dietary and other risk factors for the development of ER negative versus ER positive breast cancer.

(2) A detailed analysis of the gene expression and LOH signatures of "basal-like" breast cancers, an important subclass of ER negative breast cancer.

(3) Elucidation of the role of PI3K signaling in ER negative breast cancer.
(4) Defined a putative role for psoriasin in breast tumor progression.
(5) Progress in the analysis of the role of NFkappaB signaling in ER negative breast cancer.
(6) Exploration of the potential utility of ductal lavage as a technology for the assessment of biomarker modulation in breast cancer prevention studies targeting PPARγ.

Project 1. Epidemiology and Pathologic Precursors-

This project has made very considerable progress since the inception of the grant towards the goal of testing the hypothesis that the causes of ER-negative breast cancer are distinct from ER-positive breast cancer. This has resulted in a number of important contributions in this area that have been published in the following papers.

1. Chen WY, Hankinson SE, Schnitt SJ, Rosner BA, Holmes MD, Colditz GA. Association of hormone replacement therapy to estrogen and progesterone receptor status in invasive breast carcinoma. Cancer. 2004 Oct 1;101(7):1490-500.

We analyzed 2548 malignancies that developed among eligible postmenopausal women in that cohort between 1980 and 2000 and for which data on ER and PR status were available. RESULTS: Compared with women who had never used HRT, current long-term users of HRT were more likely to develop ER-positive/PR-positive breast carcinoma (multivariate risk ratio [RR], 1.80; 95% confidence interval [CI], 1.52-2.12) but were not any more likely to develop ER-negative/PR-negative disease (multivariate RR, 1.00; 95% CI, 0.72-1.39). This effect grew stronger with increasing duration of current HRT use and was also more pronounced among women with body mass index < 25 kg/m2. Furthermore, the association between HRT use and ER-positive/PR-positive disease was stronger among patients receiving combined HRT (CHRT) regimens, which included estrogen and progesterone, than among users of estrogen alone (ERT). CONCLUSIONS: The finding that current users of HRT were more likely to develop ER-positive/PR-positive tumors than they were to develop ER-negative/PR-negative ones suggests that both endogenous and exogenous hormonal factors may influence breast tumor characteristics. In analyses of the effects of hormonal factors on breast tumor development, ER-positive/PR-positive tumors and ER-negative/PR-negative tumors should be considered separately from each other.

2. Colditz GA, Rosner BA, Chen WY, Holmes MD, Hankinson SE. Risk factors for breast cancer according to estrogen and progesterone receptor status. J Natl Cancer Inst. 2004 Feb 4;96(3):218-28.

We conducted a prospective evaluation of risk factors for breast cancer classified according to receptor status. METHODS: During 1 029 414 person-years of follow-up of 66 145 women participating in the Nurses' Health Study from 1980 through 2000, we identified 2096 incident cases of breast cancer for which information on ER/PR status was available: 1281 were ER+/PR+, 318 were ER+/PR-, 80 were ER-/PR+, and 417 were ER-/PR-. RESULTS: We observed statistically significant heterogeneity among the four ER/PR categories for some risk factors (age, menopausal status, body mass index [BMI] after menopause, the one-time adverse effect of first pregnancy, and past use of postmenopausal hormones) but not for others (benign breast disease, family history of breast cancer, alcohol use, and height). The one-time adverse association of first pregnancy with incidence was present for PR- but not for PR+ tumors after controlling for ER status (P =.007). However, the association of BMI after menopause with incidence was present for PR+ but not PR- tumors (P =.005). Statistically significant differences in the incidence of ER+ and ER- tumors were seen with age, both before and after menopause (P =.003), and with past use of postmenopausal hormones (P =.01). Area under the receiver operator characteristic curve, adjusted for age, was 0.64 (95% confidence interval [CI] = 0.63 to 0.66) for ER+/PR+ tumors and 0.61 (95% CI = 0.58 to 0.64) for ER-/PR- tumors. CONCLUSIONS: Incidence rates and risk factors for

breast cancer differ according to ER and PR status. Thus, to accurately estimate breast cancer risk, breast cancer cases should be divided according to the ER and PR status of the tumor.

3. Missmer SA, Eliassen AH, Barbieri RL, Hankinson SE. Endogenous estrogen, androgen, and progesterone concentrations and breast cancer risk among postmenopausal women. J Natl Cancer Inst. 2004 Dec 15;96(24):1856-65.

Blood samples were prospectively collected during 1989 and 1990. Among eligible postmenopausal women, 322 cases of breast cancer (264 invasive, 41 in situ, 153 estrogen receptor [ER]-positive and progesterone receptor [PR]-positive [ER+/PR+], and 39 ER-negative and PR-negative [ER-/PR-] disease) were reported through June 30, 1998. For each case subject, we matched two control subjects (n = 643). RESULTS: We observed a statistically significant direct association between breast cancer risk and the level of both estrogens and androgens. When we restricted the analysis to case subjects with ER+/PR+ tumors and compared the highest with the lowest fourths of plasma hormone concentration, we observed an increased risk of breast cancer associated with estradiol (relative risk [RR] = 3.3, 95% confidence interval [CI] = 2.0 to 5.4), testosterone (RR = 2.0, 95% CI = 1.2 to 3.4), androstenedione (RR = 2.5, 95% CI = 1.4 to 4.3), and dehydroepiandrosterone sulfate (RR = 2.3, 95% CI = 1.3 to 4.1). In addition, all hormones tended to be associated most strongly with in situ disease. CONCLUSION: Circulating levels of sex steroid hormones may be most strongly associated with risk of ER+/PR+ breast tumors.

4. Tworoger, S. S., Eliassen, A. H., Rosner, B., Sluss, P., and Hankinson, S. E. (2004). Plasma prolactin concentrations and risk of postmenopausal breast cancer. Cancer Res 64, 6814-6819.

Prolactin is important in human breast development, and substantial laboratory and in vitro data suggest a role in mammary carcinogenesis. Therefore, we conducted a prospective case-control study nested within the Nurses' Health Study cohort to examine, in detail, the association between plasma prolactin concentrations and postmenopausal breast cancer by cancer invasiveness, estrogen receptor/progesterone receptor status, and other subject characteristics, including postmenopausal hormone use. Blood samples were collected from 1989 to 1990 and prolactin was measured by microparticle enzyme immunoassay. The analysis included 851 cases of postmenopausal breast cancer diagnosed after blood collection and before June 2000, in which there were one or two controls (n = 1,275) matched on age, postmenopausal hormone use, fasting status, and time of day and month of blood collection. Prolactin was associated with a modestly increased risk of postmenopausal breast cancer [relative risk, top versus bottom quartile, 1.34; 95% confidence interval (CI), 1.02–1.76; P-trend = 0.01]. The association differed by estrogen receptor/progesterone receptor status (P-heterogeneity = 0.03). The relative risk was 1.78 (95% CI, 1.28, 2.50; P-trend < 0.001) for estrogen receptor+/progesterone receptor+, 0.76 (95% CI, 0.43, 1.32; P-trend = 0.28) for estrogen receptor–/progesterone receptor–, and 1.94 (95% CI, 0.99, 3.78; P-trend = 0.12) for estrogen receptor+/progesterone receptor– breast cancers. Associations generally were similar for ductal and lobular carcinomas (P-heterogeneity = 0.43) and by tumor size (P-heterogeneity = 0.24). Among estrogen receptor+/progesterone receptor+ cancers, the association did not significantly differ by postmenopausal hormone use, years between blood draw and diagnosis, or after adjustment for estradiol (relative risk, 1.93; 95% CI, 1.16, 3.22; P-trend = 0.01). Our prospective data suggest that plasma prolactin concentrations are associated with an increased risk of postmenopausal breast cancer, particularly for estrogen receptor+/progesterone receptor+ cancers, and independently of estradiol.

5. Tworoger, S. S., Missmer, S. A., Barbieri, R. L., Willett, W. C., Colditz, G. A., and Hankinson, S. E. (2005). Plasma sex hormone concentrations and subsequent risk of breast cancer among women using postmenopausal hormones. J Natl Cancer Inst 97, 595-602.

BACKGROUND: Sex hormone concentrations are associated with breast cancer risk among women not using postmenopausal hormones (PMH); however, whether a relationship exists among PMH users is unknown. Therefore, we conducted a prospective, nested case-control study within the Nurses' Health

Study (NHS) cohort to examine the association between plasma sex hormone concentrations and postmenopausal breast cancer among women using PMH at blood collection. METHODS: Blood samples were collected from 1989 to 1990. During follow-up through May 31, 2000, 446 women developed breast cancer and were matched by age, date and time of day of blood collection, and fasting status to 459 control subjects (PMH users) who did not develop cancer. We used conditional logistic regression to estimate relative risks (RRs) and 95% confidence intervals (CIs). We compared hormone concentrations of the 459 control subjects with those of 363 postmenopausal NHS participants not taking PMH. All statistical tests were two-sided. RESULTS: PMH users had statistically significantly higher estradiol, free estradiol, sex hormone-binding globulin, and testosterone, and lower free testosterone concentrations than non-PMH users. Among PMH users, we found modest associations with breast cancer risk when comparing the highest versus lowest quartiles of free estradiol (RR = 1.7, 95% CI = 1.1 to 2.7; P(trend) = .06), free testosterone (RR = 1.6, 95% CI = 1.1 to 2.4; P(trend) = .03), and sex hormone-binding globulin (RR = 0.7, 95% CI = 0.5 to 1.1; P(trend) = .04), but not of estradiol or of testosterone. However, estradiol and free estradiol were statistically significantly positively associated with breast cancer risk among women older than 60 years (RR = 2.8, 95% CI = 1.5 to 5.0; P(trend) = .002 and 2.6, 95% CI = 1.4 to 4.7; P(trend) = .001, respectively) and among women with a body mass index of less than 25 kg/m2 (RR = 1.8, 95% CI = 1.1 to 3.1, P(trend) = .01 and 2.4, 95% CI = 1.4 to 4.0, P(trend) = .003, respectively). CONCLUSION: Although women using PMH have a different hormonal profile than those not using PMH, plasma sex hormone concentrations appear to be associated with breast cancer risk among PMH users.

6. Zhang, S.M., Hankinson, S.E., Hunter, D.J., Giovannucci, E.L., Colditz, G.A., and Willett, W.C. (2005). Folate intake and risk of breast cancer characterized by hormone receptor status. Cancer Epidemiol Biomarkers Prev 14(8), 2004-2008.

Folate plays an important role in DNA methylation, and aberrant methylation of the estrogen receptor (ER) gene may be related to the loss of ER gene expression in breast tumors. Thus, deficient folate status has been hypothesized to be associated primarily with ER gene-negative breast tumors, but data relating folate intake to breast cancer risk according to ER status are sparse. We conducted a prospective cohort analysis of folate intake among 88,744 women in the Nurses' Health Study who completed a food frequency questionnaire in 1980 and every 2 to 4 years thereafter. During 20 years of follow-up, 2,812 ER+ and 985 ER- invasive breast cancer cases were documented. Higher total folate intake was significantly associated with lower risk of developing ER- but not ER+ breast cancer; the multivariable relative risks (RR) and 95% confidence intervals (95% CI) comparing the highest to the lowest quintile were 0.81 (0.66-0.99) for ER- tumors and 1.00 (0.89-1.14) for ER+ tumors. The inverse association between total folate intake and ER- breast cancer was mainly present among women consuming at least 15 g/d of alcohol (multivariable RR, 0.46; 95% CI,=0.25-0.86; top versus bottom quintile). These findings support the hypothesis that higher folate intake reduces the risk of developing ER- breast cancer. Ensuring adequate folate intake seems particularly important for women at higher risk of breast cancer because of alcohol consumption.

Project 2. Genomic Fingerprinting-

Accomplishments:

1. Described the genotype of an aggressive form of estrogen receptor (ER)-negative breast cancer, called the "basal-like" tumor. This included a genome wide map of allelic loss, definition of signature chromosomal loss, and the frequencty of allelic loss in the basal-like tumor.

2. Described a particular defect in X-chromosome gene dosage found in the basal-like, ER-negative breast cancers.

3. Contributed to the subclassification of invasive breast cancer by gene expression array, and made our datasets available to the research community.

4. Examined the contribution of BRCA1 gene methylation to the basal-like breast cancer, finding that BRCA1 is probably normally expressed and regulated in these tumors.

Provide a profile for ER-negative cancers using Affymetrix U133 expression arrays and high-density SNP arrays.

This work was done with cancer specimens collected by the SPORE in Breast Cancer at the Brigham and Women's Hospital, using funds from the SPORE and from private sources within the Women's Cancers Program of the Dana-Farber Cancer Institute. Affymetrix U133 GeneChips and high-density SNP arrays have been acquired for this work using private funds from the Women's Cancers Program and from the Brigham and Women's Hospital. The Department of Defense Center of Excellence provided labor and other supplies to accomplish this goal. The progress of this project is reflected in the following original publications (#3 is still under review and should be kept confidential).

1. Wang ZC, Lin M, Wei L-J, Li C, Miron A, Lodeiro G, Harris L, Ramaswamy S, Tanenbaum DM, Meyerson M, Iglehart JD, Richardson A: Loss of heterozygosity and its correlation with expression profiles in subclasses of invasive breast cancers. Cancer Res. 64: 64-71, 2004.

Gene expression array profiles identify subclasses of breast cancers with different clinical outcomes and different molecular features. The present study attempted to correlate genomic alterations (loss of heterozygosity; LOH) with subclasses of breast cancers having distinct gene expression signatures. Hierarchical clustering of expression array data from 89 invasive breast cancers identified four major expression subclasses. Thirty-four of these cases representative of the four subclasses were microdissected and allelotyped using genome-wide single nucleotide polymorphism detection arrays (Affymetrix, Inc.). LOH was determined by comparing tumor and normal single nucleotide polymorphism allelotypes. A newly developed statistical tool was used to determine the chromosomal regions of frequent LOH. We found that breast cancers were highly heterogeneous, with the proportion of LOH ranging widely from 0.3% to >60% of heterozygous markers. The most common sites of LOH were on 17p, 17q, 16q, 11q, and 14q, sites reported in previous LOH studies. Signature LOH events were discovered in certain expression subclasses. Unique regions of LOH on 5q and 4p marked a subclass of breast cancers with "basal-like" expression profiles, distinct from other subclasses. LOH on 1p and 16q occurred preferentially in a subclass of estrogen receptor-positive breast cancers. Finding unique LOH patterns in different groups of breast cancer, in part defined by expression signatures, adds confidence to newer schemes of molecular classification. Furthermore, exclusive association between biological subclasses and restricted LOH events provides rationale to search for targeted genes.

2. Matros E., Wang Z.C., Lodeiro G., Miron A., Iglehart J.D., Richardson A.L.. BRCA1 expression in sporadic breast tumors: relation to proliferation, promoter methylation and gene expression profiles. *Breast Cancer Res Treat* 91: 179-86, 2005.

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4. Richardson A.L., Wang Z.C., Lu X., Miron A., Iglehart J.D., Livingston D.M., Ganesan, S. Specific contributions by X chromosomal abnormalities to basal-like, human breast cancer. *Cancer Cell*, submitted, 2005.

Breast cancers in women carrying a germ line BRCA1 mutation reveal defects in the maintenance of a normal, inactive X chromosome (Xi) and are phenotypically similar to sporadic basal-like cancers (BLC). Most BLC also lack a normal Xi-associated heterochromatic superstructure and Xi CpG island methylation. Duplication of the active X chromosome and loss of Xi were present in almost half of BLC cases tested. Others contained bi-parental but non-heterochromatinized X chromosomes or gains of additional, non-heterochromatinized X chromosomal DNA. These abnormalities, which were rare in non-basal-like, high-grade breast carcinomas, were associated with over-expression of a small subset of X chromosomal genes. These results suggest that X chromosome abnormalities contribute to the pathogenesis of BLC, both inherited and sporadic.

Women harboring a germ line mutation in BRCA1 experience a 50-80% lifetime risk of developing breast cancer. The breast cancers that arise in these individuals are phenotypically similar to sporadic basal-like cancers (BLC), both by histology and gene expression profiling. Sporadic BLC are high grade, aggressive breast cancers with wild-type BRCA1 genes, the pathogenesis of which is unknown. Recently, it was shown that BRCA1-associated cancers reveal a defect in the maintenance of a normal, inactive X chromosome (Xi) and that BRCA1 actively contributes to the maintenance of Xi heterochromatinization. The study reported here demonstrates that sporadic BLC also experience such an Xi defect, strongly suggesting that a major defect in Xi heterochromatinization contributes to the pathogenesis of both inherited, BRCA1-deficient and the more common, sporadic BLC.

Project 3. Role of Growth Factor Signaling-

Progress:

We have identified and/or characterized multiple genes that might contribute to the development of ER-negative tumors:

1) Phosphatidylinositol 3'-kinase alpha (PIKC3A):

Mutations in PIK3CA have been identified in approximately 25-30% of ER-negative breast tumors. To examine the functional consequences of expression of these proteins in human breast epithelial cells, we introduce the two most common isoforms into MCF-10A cells and examined the behavior of these proteins in a variety of cellular assays. MCF-10A cells expressing the mutant PIK3CA exhibited increased PI3K activity, anchorage-independent proliferation, growth factor-independent proliferation, and protection from anoikis. Furthermore, PIK3CA mutations induced abnormal mammary acinar morphogenesis in 3D basement membrane cultures and conferred increased resistance to paclitaxel. These results suggest that cancer-associated mutations in PIK3CA may significantly contribute to breast cancer pathogenesis and resistance to chemotherapy and represent attractive targets for therapeutic inhibition.

Zhao, J. J., Gjoerup, O. V., Subramanian, R. R., Cheng, Y., Chen, W., Roberts, T. M., and Hahn, W. C. (2003). Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. Cancer Cell 3, 483-495.

Isakoff, S.J., Engelman, J.A., Irie, H.Y., Luo, J., Brachmann, S.M., Pearline, R.V., Cantley, L., and Brugge, J.S., Breast Cancer Associated *PIK3CA* Mutations Are Oncogenic in Mammary Epithelial Cells, in press.

2) **p53 related protein p63**

p63 is known to play a critical role in the development of stratified epithelia and its derivative tissues and its expression is elevated in a variety of tumors including a subclass of ER-negative breast tumors; however little is known about the specific cellular programs that are regulated by this transcription factor. We utilized the normal breast epithelial cell line MCF-10A as model system to investigate cellular processes regulated by p63. Overexpression of p63 led to a dramatic hyperproliferativce phenotype and an increase in cell adhesion. By characterizing transcriptional profiles and cellular effects following specific loss and gain of p63 function we have defined a vital role for p63 in cellular adhesion. Knockdown of endogenous p63 expression in MCF-10A cells caused downregulation of cell adhesion-associated genes and proteins, cell detachment and anoikis. Similar results were obtained in primary mammary epithelial cells and keratinocytes. Conversely, increased expression of either TAp63γ or ΔNp63α isoforms upregulated genes encoding cell adhesion molecules, increased cellular adhesion to exogenous ECM and conferred resistance to anoikis. Moreover anoikis induced by p63 loss was rescued by signaling downstream of β4 integrin. Our results implicate p63 as a key regulator of the cellular program of adhesion and survival in basal cells of the mammary gland and other stratified epithelial tissues. This function of p63 could play a role in p63's function in tumor development.

Carroll, D.K, Carroll" J.S., Leong' C-O, Cheng, F.,, Brown' M. , Mills,A.A., Brugge, J.S. and Ellisen' L.W. p63 Regulates an Adhesion Program and Cell Survival in Epithelial Cell, submitted.

3) PDEF

The Ets transcription factor, PDEF, was identified in a unique retroviral mediated motility screen in mammary epithelial cells. PDEF is overexpressed in the epithelial cells of >75% of breast tumors ranging from early to late stages of carcinoma. Co-expression of PDEF with the receptor tyrosine kinases (RTKs) ErbB2 and CSF-1R, enhanced motility, induced invasion in 3D cultures, and promoted growth on soft agar. Mutation of an optimal MAPK phosphorylation site abolished the migratory and invasive activity of PDEF, suggesting that Ras/MAPK regulates PDEF activity.. Therefore, PDEF may serve as a biomarker for early detection of aggressive tumors and a molecular target of RTK activated metastatic carcinomas.

Gunawardane R. N., Sgroi D. C., Wrobel C. N., Koh E., Daley G. Q., and Brugge J.S. , A novel role for the ETS transcription factor, PDEF, in epithelial cell migration and invasion. Cancer Res. *In press*..

4) IGF-1 Receptor and the Akt protein kinase

Akt/PKB kinases are activated by growth factors and regulate pleiotropic cellular activities. Here we provide evidence for isoform-specific, positive and negative roles of Akt1 and Akt2 in regulating growth factor-stimulated phenotypes in breast epithelial cells. The morphological, hyperproliferative and anti-apoptotic activities induced by insulin-like growth factor-I receptor (IGF-IR) hyperstimulation were reversed by Akt2 downregulation. In contrast, Akt1 downregulation promoted dramatic neomorphic effects in IGF-IR hyperstimulated cells, consistent with an epithelial-mesenchymal transition (EMT) and enhanced cell migration induced by IGF-I or EGF stimulation. The phenotypic effects of Akt1 downregulation were accompanied by enhanced ERK activation, which contributed to the induction of migration and EMT. Interestingly, downregulation of Akt2 suppressed the EMT-like morphological conversion induced by Akt1 downregulation in IGF-IR overexpressing cells and inhibited migration in EGF stimulated cells. These results highlight the distinct functions of Akt isoforms in regulating the outcome of growth factor stimulation and the importance of Akt1 in cross-regulation of two major signaling pathways.

Irie . H.Y.,. Pearline R. V, Grueneberg D. ,. Hsia M,. Ravichandran P, Brugge, J. S.

Distinct Roles of Akt1 and Akt2 in Regulating Cell Migration and Epithelial-Mesenchyme Transition, submitted

INTRODUCTION

We identified HID5/psoriasin based on SAGE (Serial Analysis of Gene Expression) analysis of normal mammary epithelial cells and in situ and invasive breast carcinomas as one of the transcripts most abundantly and specifically expressed in DCIS (ductal carcinoma in situ) cells. HID5/psoriasin is a small calcium binding protein that belongs to the family of S100 proteins, but it's physiological function and role in breast cancer are unknown. However, several other members of the S100 family have been implicated in breast tumor progression including S100A2 and S100A4 (1, 2). We and others have demonstrated that the expression of HID5/psoriasin is highest in high grade DCIS, and although it is somewhat decreased in invasive tumors, a significant fraction of ER negative tumors express it (3-6). More recently the overexpression of psoriasin in MDA-MB-231 cells has been reported to enhance tumorigenicity in vivo and also to have a modest effect on the growth and invasive behavior of the cells in vitro (7-12). Correlating with this the expression of psoriasin in ER negative tumors correlates with poor outcome (7-12).

The goal of this project is to characterize the function of the HID5/psoriasin gene and its role in breast cancer. In order to achieve this we proposed three specific aims in the original application: (1) Analyze the role of HID5/psoriasin in the regulation of mammary cell growth and survival, (2) Analyze HID5/psoriasin expression in primary tumors using tissue microarrays, and (3) Evaluate HID5/psoriasin as a potential breast cancer diagnostic marker. We have made significant progress relevant to all three aims as described in detail below.

BODY

Specific Aim 1. Analyze the role of HID5/psoriasin in the regulation of mammary cell growth and survival

Generation of cells expressing psoriasin shRNA

Previous studies analyzing the function of psoriasin in breast cancer used a cell line exogenously overexpressing psoriasin (11). Since exogenous overexpression can lead to non-physiologic expression levels and the results could be influenced by the choice of cell type, eliminating the expression of a gene in the cell that expresses it endogenously is more likely to yield physiologically relevant results. Thus, in order to dissect the functional relevance of psoriasin expression in breast cancer, we derived stable clones from the MDA-MB-468 human breast cancer cell line that expressed control or psoriasin shRNA (short hairpin RNA). We designed three different shRNAs corresponding to different areas of the psoriasin mRNA. Pso-RNAi-1 targets the 5' untranslated region, while Pso-RNAi-2 and Pso-RNAi-3 target the proximal and distal portion of the coding region, respectively. In addition, we also designed shRNAs corresponding to CXCL12 and CXCL14 as controls, since neither of these genes are expressed in MDA-MB-468 cells at levels detectable by northern blot analysis (13). All of these shRNAs were sublconed into the pLKO-puro construct and used for the establishment of stable clones. The effectiveness of the shRNAs was confirmed by immunoblot analysis of cell extracts prepared from pools of cells following selection. Pso-RNAi-2 and to a lesser degree Pso-RNAi-1 dramatically downregulated psoriasin protein levels, while Pso-RNAi-3 and the control CXCL12 and CXCL14 shRNAs had no significant effect on psoriasin expression (Fig. 1*A*). Since we derived pools of stable clones in order to avoid clonal selection, we also performed immunocytochemical analysis of the cells to determine inter-cellular heterogeneity within the pools. We found that the expression of psoriasin was uniformly downregulated in the Pso-RNAi-1 and Pso-RNAi-2 cells, while the pLKO-puro and Pso-RNAi3 and control shRNA cells all expressed high levels of psoriasin (Fig. 1*B* and data not shown).

The effect of psoriasin on cell proliferation, survival, migration and invasion in vitro

Next we analyzed the association between psoriasin expression and cellular behavior *in vitro* using the above described stable pools. We found no significant difference between the proliferation of control pLKO-puro and Pso-RNAi-1-3 cells (Fig. 1*C*). Similarly there was no significant difference in the sensitivity of control and psoriasin shRNA expressing cells to various apoptosis inducing stimuli

including chemotherapeutic agents, serum and glucose deprivation, and oxidative stress induced by menadione and hydrogen peroxide (data not shown). However, we observed statistically significantly increased colony numbers in soft agar assays using the Pso-RNAi-1 (p=0.01) and Pso-RNAi-2 (p=0.0001) cells compared to controls, suggesting that downregulation of psoriasin in MDA-MB-468 cells enhances anchorage independent growth (Fig. 1*D*). The influence of psoriasin expression on cell migration and invasion was assessed using a modified Boyden chamber assay (14). There was also a consistent and significant increase in the motility and invasion of cells with decreased psoriasin levels, suggesting that psoriasin may have a negative effect on cell migration and invasion (Fig. 1*E* and *F*). Interestingly overexpression of psoriasin in MDA-MB-231 cells was reported to increase cell proliferation, motility, and invasion, although these effects were very modest *in vitro* (1.2-1.5 fold difference compared to controls) (11). Similarly overexpression of S100A4 in mouse mammary epithelial cells derived from neu transgenic mice leads to increased motility and invasion, but this was not correlated with increased proteolytic degradation of the extracellular matrix (15). Although we do not know the reason for the apparent discrepancy between our and previously reported results,

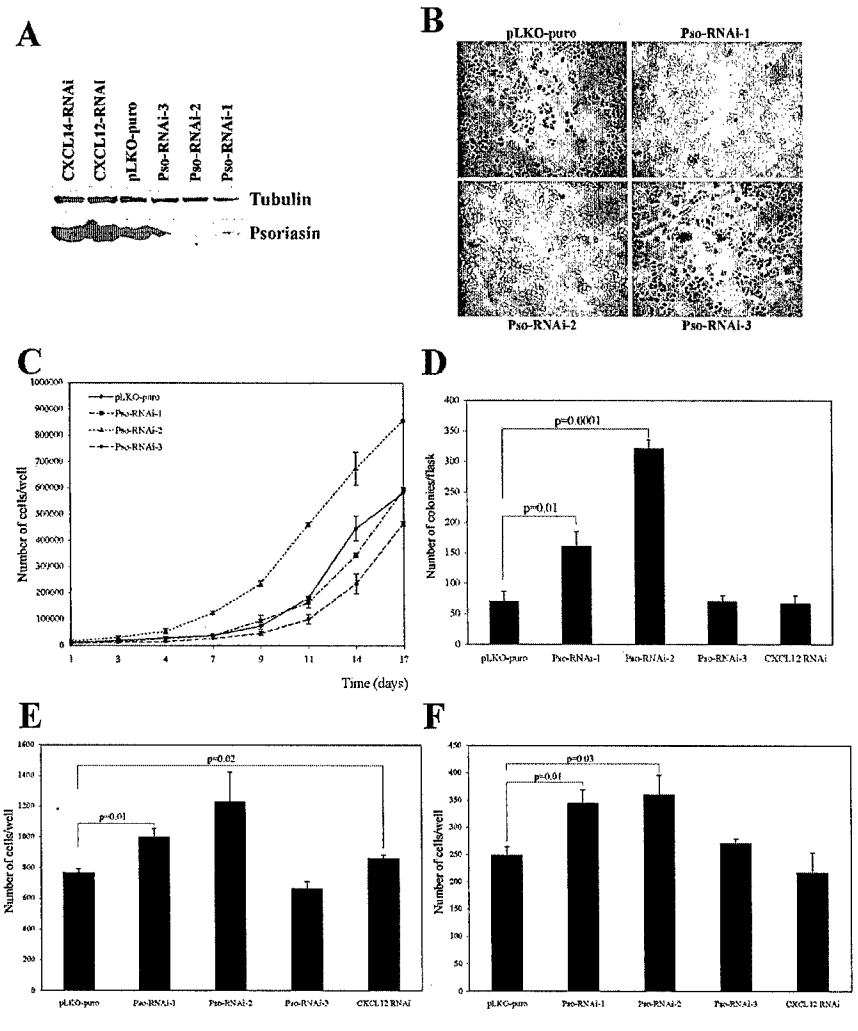

Figure 1. Generation and characterization of MDA-MB-468 cells expressing psoriasin shRNAs. ***A,*** Immunoblot analysis of psoriasin expression in control MDA-MB-468 pLKO-puro, three different psoriasin shRNA (Pso-RNAi-1, 2, 3), and two control shRNA (CXCL12 and CXCL14) expressing cells. The extracts were probed for β-tubulin expression to indicate equal loading. ***B,*** Immunocytochemical analysis of psoriasin expression in control MDA-MB-468 pLKO-puro and three different psoriasin shRNA expressing cells. ***C,*** Results of a representative *in vitro* proliferation assay demonstrating no statistically significant differences among the growth rates of control pLKO-puro and Pso-RNAi-1, 2, 3 cells. ***D,*** Downregulation of psoriasin expression leads to increased soft agar growth in MDA-MB-468 cells. Quantitative summary of the result of a representative soft agar assay with mean colony counts, p values of statistically significant differences [Pso-RNAi-1 (p=0.01) and Pso-RNAi-2 (p=0.0001) cells compared to controls] and standard deviations plotted. ***(E)*** and invasion ***(F)***. Columns represent means and ±SD of a representative experiment performed in triplicate. p values of statistically significant differences compared to pLKO-puro cells are indicated.

the use of different cell types and exogenous overexpression instead of downregulation of endogenous prosiasin expression could explain the observed differences in the *in vitro* behavior of the cells.
During breast tumor development one of the most critical transitions is the progression from *in situ* to invasive carcinoma. Immunohistochemical analyses of adjacent DCIS and invasive breast tumors demonstrate decreased psoriasin expression in the invasive areas suggesting that downregulation of psoriasin may play a role in the *in situ* to invasive carcinoma progression (4, 5, 10) potentially by relieving psoriasin mediated inhibition of migration and invasion of the cancer cells. Our results demonstrating increased migration, invasion, and soft agar growth following downregulation of psoriasin in MDA-MB-468 cells support this hypothesis. However, since the MDA-MB-468 cells were derived from a metastatic breast carcinoma our results may not be directly interpretable for DCIS tumors. Thus, understanding the function of psoriasin in the *in situ* to invasive carcinoma progression requires further studies.

The effect of psoriasin on in vivo tumorigenicity in nude mice

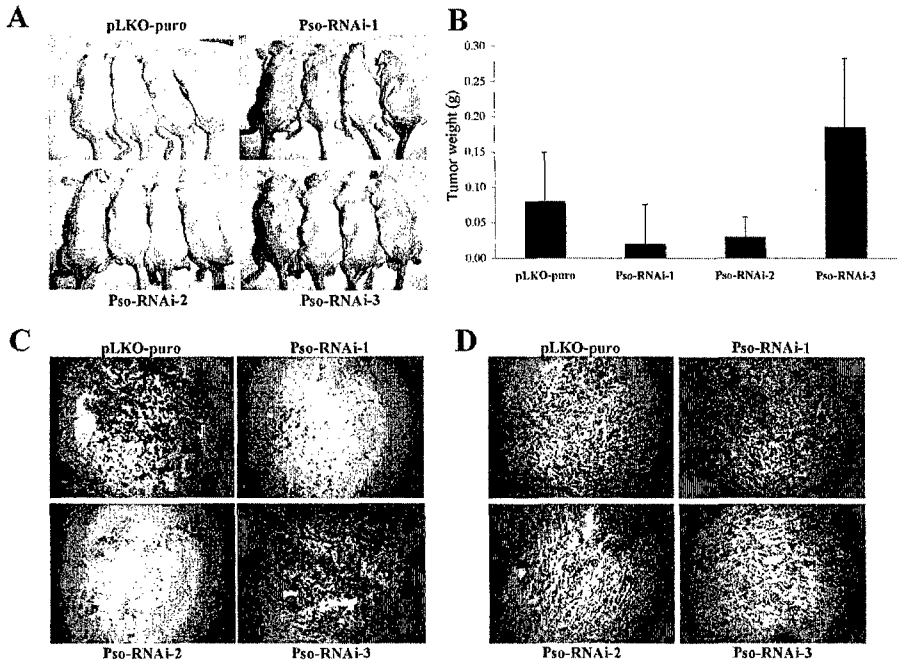

Figure 2. The effect of psoriasin expression on tumor growth in nude mice. ***A,*** Representative mice at 10 weeks after injection from each experimental group. Compared to the control pLKO-puro and Pso-RNAi-3 groups several mice in the Pso-RNAi-1 and Pso-RNAi-2 groups lacked visible tumors. ***B,*** Mean tumor weight ±SD of each experimental group of mice combined from two independent experiments (20 injections total). In each experiment five mice/group were used and each mouse was injected at two sites. Downregulation of psoriasin expression in the Pso-RNAi-1 and Pso-RNAi-2 cells significantly diminished tumor size. p values of statistically significant differences compared to tumors generated from pLKO-puro cells are indicated. ***C,*** Immunohistochemical analysis of psoriasin expression in the xenografts confirmed the stability and effectiveness of the shRNAs. ***D,*** Immunohistochemical analysis of MIB-1 (Ki67) expression in the xenografts did not reveal significant differences in the proliferation rates of the cells, consistent with the results of the *in vitro* growth assays.

To determine if psoriasin expression also correlates with invasion and cell growth in vivo, control pLKO-puro and Pso-RNAi-1, 2, 3 cells were injected subcutaneously into the flank of female nude mice, and tumor growth and metastasis were assessed. Control pLKO-puro and Pso-RNAi-3 (ineffective RNAi) cells generated tumors at nearly all injection sites (17/20 and 9/10, respectively) after 10 weeks of growth and these tumors were first noted at 2-3 weeks after injection. In mice injected with Pso-RNAi-1 and Pso-RNAi-2 cells several injection sites did not result in tumors or developed tumors that were barely measurable (only 6/20 injections resulted in measurable size tumors). These tumors also appeared somewhat later after injection (3-4 weeks) and grew much slower than the controls (Fig. 2*A*). Analysis of tumor weight at 10 weeks after injection revealed that downregulation of psoriasin in the Pso-RNAi-1 and Pso-RNAi-2 cells dramatically

reduced tumorigenicity (Fig. 2*B*). The mean tumor weight combined from two independent experiments in the control pLKO-puro group was 0.19g, while in Pso-RNAi-1 and Pso-RNAi-1 groups was 0.05 and 0.07g, respectively. Wilcoxon Rank sum test determined that both of these experimental groups were statistically significantly different from controls (p=0.05 and p=0.05 for Pso-RNAi-1 and Pso-RNAi-2, respectively). Tumors derived from cells with the ineffective psoriasin (Pso-RNAi-3) or irrelevant control (CXCL12) shRNA did not differ significantly from the pLKO-puro controls (Fig. 2*A, B*, and data not shown).

Microscopic examination of the tumors resulting from the different experimental groups did not reveal differences in histologic appearance, although the larger tumors in the control and Pso-RNAi-3 groups tended to have more extensive necrotic areas (data not shown). Immunohistochemical analysis of psoriasin expression in the tumors confirmed the stable downregulation of psoriasin in the Pso-RNAi-1 and Pso-RNAi-2 cells and uniformly high psoriasin levels in pLKO-puro and Pso-RNAi-3 cells (Fig. 2*C*). Staining for MIB1 (Ki67) expression, a marker of proliferating cells, determined that all tumors were very highly proliferative irrespective of the expression of psoriasin (Fig. 2*D*). This result correlates with the results of our *in vitro* cell proliferation assays that also did not reveal differences among the different groups (Fig. 1*C*).

We also assessed if the expression of psoriasin influences metastatic behavior, but we did not see grossly evident lung and other major organ metastases in any of the experimental groups. The MDA-MB-468 cell line is not highly metastatic and the tumors were only allowed to grow to a relatively small size and short time (10 weeks). However, analysis of lung micrometastases using RT-PCR and primers specific for human DNA (hHPRT gene) revealed micrometastasis in all mice in the control, but not in the Pso-RNAi-1 and Pso-RNAi-2 groups (data not shown). This result is in agreement with prior studies demonstrating increased number of abdominal lymph node metastases in mice injected with psoriasin overexpressing MDA-MB-231 cells compared to controls (11).

The effect of psoriasin on the expression of VEGF and MMP13

In order to begin to dissect the molecular bases of the observed *in vitro* and *in vivo* phenotypic differences between control and psoriasin shRNA expressing cells, we analyzed the expression of genes involved in angiogenesis and invasion. Previous data suggested an association between psoriasin expression and VEGF and MMP13 mRNA levels in human breast cancer cells (11). Specifically, exogenous overexpression of psoriasin in MDA-MB-231 cells led to increased AP-1 transcriptional activity and subsequent increase in the RNA levels of two of its target genes VEGF and MMP13 (15). In order to determine if such association also exists in our experimental system, we analyzed the expression of VEGF and MMP13 by real-time RT-PCR in exponentially growing control and Pso-RNAi-2 cells cultured *in vitro*. We also analyzed the expression of psoriasin itself as a control for the assay and β-actin as a reference gene. We confirmed the downregulation of psoriasin mRNA in the Pso-RNAi-2 cells compared to control pLKO-puro cells validating the reliability of the assay and also the stability of the clones and the efficiency of shRNA (Fig. 3*A*). Correlating with previous data, we found statistically significant (p=0.02) downregulation of VEGF in the Pso-RNAi-2 cells compared to controls, however, the expression of MMP13 was statistically significantly (p=0.02) increased (Fig. 3*A*). Although the up-regulation of MMP13 is the opposite of that expected based on prior results, it is consistent with our observation that the invasiveness of the Pso-RNAi-2 cells is increased compared to controls (Fig. 1*F*). To better understand the contribution that the induction of MMP-13 expression may make to the migration and invasion of the MDA-MB-468 cells, we examined the effect of a specific inhibitor of MMP-13 in the in vitro migration/invasion assay (16). We found that the MMP-13 inhibitor had no appreciable effect on the migration of the pLKO-puro or Pso-RNAi-2 cells (Fig. 3*B*). However, inhibition of MMP-13 led to a significant (p=0.003) reduction in the invasive capacity of the Pso-RNAi-2 cells while having little effect on that of the pLKO-puro cells (Fig. 3*B*). This provides further support for the hypothesis that upregulation of MMP-13 plays a role in the increased invasive ability of the Pso-RNAi-2 cells. The differences observed between the present results and those of a prior study may be

attributable to differences in the techniques utilized; the published report overexpressed psoriasin in a cell line that does not normally make detectable levels of the protein. In contrast, here shRNA was used to knockdown psoriasin expression in a cell line with high endogenous protein high levels. In general a loss-of-function approach felt to provide a more physiological read out of a protein's function than that derived from overexpression.

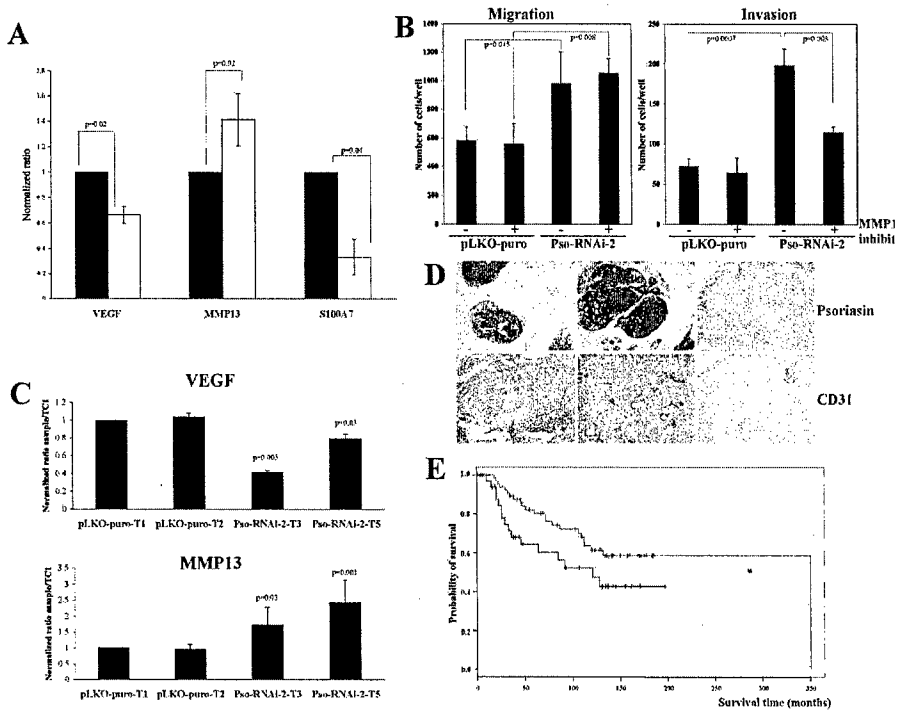

Figure 3. The effect of psoriasin levels on the expression of VEGF and MMP13. ***A,*** Real-time PCR analysis of the expression of psoriasin, VEGF, and MMP13 in control pLKO-puro (black bars) and Pso-RNAi-2 (white bars) cells. ***B,*** The effect of a specific MMP13 inhibitor (CL-82198) on the migration and invasion of control (pLKO-puro) and psoriasin RNAi (Pso-RNAi-2) cells. ***C,*** Real-time PCR analysis of the expression of VEGF and MMP13 in two representative independent tumors derived from control pLKO-puro (T1 and T2) and Pso-RNAi-2 (T3 and T5) cells. ***D,*** Immunohistochemical analysis of CD31 endothelial cell marker expression to assess the degree of angiogenesis in a representative set of three ductal carcinomas. The same tumors were also analyzed for the expression of psoriasin. There was a positive correlation between CD31 and psoriasin expression. The top two tumors demonstrate high psoriasin expression and high blood vessel density, while the psoriasin negative tumor in the bottom panel is largely CD31 negative. ***E,*** Relationship between psoriasin expression and clinical outcome. Kaplan-Meier survival curve depicting association between psoriasin expression and death due to breast cancer in 108 patients. Patients with psoriasin positive tumors (red line) had shorter disease specific survival than patients whose tumors were negative for psoriasin (green line), but this was of borderline statistical significance (p=0.05).

To determine if the differences between control and Pso-RNAi-2 cells in VEGF and MMP13 levels are maintained during *in vivo* tumor growth, we repeated the real-time PCR analysis using two representative tumor samples from both control and Pso-RNAi-2 groups. Analysis of the tumor samples gave identical results to what we found in the cells cultured *in vitro*. Tumors generated from the Pso-RNAi-2 cells demonstrated statistically significantly decreased VEGF and increased MMP13 mRNA levels (Fig. 3*C*).

Thus, the decreased VEGF levels could be partially responsible for the decreased tumor take and growth observed following the injection of the Pso-RNAi-2 cells. Interestingly psoriasin itself appears to be regulated by ROS (reactive oxygen species), since its expression is increased in cells treated with a low concentration of hydrogen peroxide in MCF10A mammary epithelial cells (17). Moreover, the induction of its expression by detachment from the extracellular matrix can be completely eliminated by anti-oxidant treatment or bcl-2 overexpression or by inhibiting NFkB activation (17). Since hypoxic conditions lead to increased ROS levels, psoriasin may play a role in the upregulation of VEGF and induction of angiogenesis under these conditions. Alternatively, psoriasin and VEGF may be regulated by the same transcriptional mechanisms. High-grade comedo DCIS tumors, which frequently overexpress psoriasin, have high rates of hypoxia, apoptosis, and necrosis potentially

causing the upregulation of psoriasin in these tumors (18). Overexpression of psoriasin subsequently may increase the levels of VEGF and enhance angiogenesis. Correlating with this hypothesis high-grade comedo DCIS is associated with increased angiogenesis (19). To further explore the physiologic function of psoriasin in human breast cancers, we analyzed its expression in microarray data from 89 invasive breast carcinomas (27) and using a k-nearest neighbor (k-NN) algorithm (20, 21) identified genes whose expression most highly correlated with the expression of psoriasin (Table 1). A surprisingly high fraction (26%) of these 50 highest ranked genes is localized to chromosome 1q, a chromosomal arm where psoriasin itself and an S100 gene cluster are located, potentially suggesting co-regulation of the genes or underlying genetic change. Gain of 1q is one of the most common and earliest genetic events in breast cancer (22). Confirming previous data demonstrating co-regulation of psoriasin with S100A9 and other S100 proteins (17), S100A9 and S100A8 were most closely correlated with psoriasin expression (Table 1). Interestingly VEGF itself was identified by this analysis further strengthening the association between psoriasin and VEGF expression. In addition, a significant fraction (26%) of the 50 genes represented mitochondrial proteins involved in energy metabolism. This suggests that these genes are coordinately regulated with psoriasin in invasive breast cancers and provides further evidence linking psoriasin to ROS and angiogenesis.

Specific Aim 2. Analyze HID5/psoriasin expression in primary tumors using tissue microarrays

Psoriasin expression in human primary breast carcinomas

The decreased VEGF levels in MDA-MB-468 cells with decreased psoriasin levels and the high expression of psoriasin in high-grade, comedo DCIS associated with increased VEGF levels and blood vessel density (19) suggested that psoriasin may play a role in angiogenesis. To determine if there is an association between psoriasin expression and angiogenesis in human breast tumors, we performed immunohistochemical analysis of psoriasin and CD31 (an endothelial cell specific marker) expression on a tissue microarray composed of 49 invasive breast carcinomas and 10 normal breast tissue and fibroadenomas. Based on this analysis we found a statistically significant ($p=0.02$) positive correlation between psoriasin expression and blood vessel density (determined by quantitating the intensity and fraction of CD31 positive cells/tissue spot). Representative psoriasin and CD31 staining results are depicted in Fig. 3*D*.

In order to analyze the relationship between psoriasin expression and clinical outcome in breast cancer, we performed immunohistochemical analysis of a tissue microarray composed of 138 cases of invasive breast tumors with clinical follow-up data. The median follow up was 138 months; a detailed description of the patient cohort has been previously reported (23). We examined the association between psoriasin expression and various tumor characteristics, including the expression of estrogen and progesterone receptors (ER and PR) and HER2, size of tumor, total number of positive lymph nodes, clinical stage, tumor grade, presence of angioinvasion, and age of patients. We found that the expression of psoriasin was statistically significantly more frequently observed in ER negative ($p=0.00006$), PR negative ($p=0.000002$), HER2 positive ($p=0.02$), and high-grade (0.001) tumors. In addition, patients with psoriasin positive tumors were more likely to be under 50 years of age ($p=0.01$). There was no statistically significant association between the expression of psoriasin and other tumor and patient characteristics (data not shown).

Analysis of time-to-recurrence suggested that patients with tumors that lacked psoriasin expression survived longer than patients with psoriasin positive tumors, but this did not reach statistical significance ($p=0.06$; Fig. 3*E*). Similarly, investigation of the association between psoriasin expression and overall survival revealed a positive association between psoriasin expression and decreased survival, but this was of borderline statistical significance ($p=0.05$). This result is consistent with previous studies demonstrating association between poor clinical outcome and psoriasin expression in invasive breast carcinomas (12).

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of psoriasin shRNA system in mammalian cells
- Characterization of the effect of psoriasin depletion on cell growth, invasion, and in vivo tumorigenicity
- Identification of MMP13 and VEGF as mediators of psoriasin's anti-invasive and pro-angiogenic effects, respectively.
- Analysis of psoriasin expression in a large set of breast tumors, correlating this with angiogenesis and clinical outcome

REPORTABLE OUTCOMES

Manuscripts:

1. Carlsson H, Yhr M, Petersson S, Collins N, Polyak K, Enerback C. Psoriasin (S100A7) and Calgranulin-B (S100A9) Induction is Dependent on Reactive Oxygen Species and is Downregulated by Bcl-2 and Antioxidants. Cancer Biol Ther. 2005; 4:

2. Krop I, März A, Carlsson H, Li X, Bloushtain-Qimron N, Hu M, Gelman R, Sabel MS, Schnitt S, Kleer CG, Enerbäck C, Polyak K. A putative role for psoriasin in breast tumor progression. Cancer Res *in press*

CONCLUSIONS

Based on our findings we propose that modulation of psoriasin expression and/or function is an important step in the progression of breast cancer from the *in situ* to invasive stage. The ability of psoriasin to negatively influence cell migration, invasion, and anchorage independent growth must be overcome during tumor progression, although the mechanism may be highly dependent on the particular molecular subtype of breast cancer. For many cancers in transition, this is accomplished via downregulation of psoriasin expression. This scenario is consistent with the observation that in many breast carcinomas the expression of psoriasin is markedly higher in DCIS compared to adjacent invasive tumors. However, in a subset of invasive cancers (e.g. high grade and/or ER negative tumors) hypoxia and ROS are dominant factors and the positive effects of psoriasin expression, particularly pro-angiogenic effects, may outweigh the negative effects or the cells may become resistant to them, and the tumor therefore retains high psoriasin expression. This hypothesis is supported by data demonstrating that psoriasin expression is induced by ROS (17) and our observation of coordinated expression of psoriasin and ROS associated genes in breast cancers. Certainly our data and prior reports indicate that the role of psoriasin in *in situ* and invasive cancer is complex and likely differs in distinct breast cancer subtypes. A full understanding of the role of psoriasin in breast cancer will thus require further studies.

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Project 5. Development of NF-κB Directed Therapy

The concept of the potential role of NF-κB-activation in human breast cancer evolved from the frequently observed Elevated level of this transcription factor in human breast cancer cells in culture, predominantly in Estrogen Receptor-negative (ER-negative) cells (**Biswas et al. PNAS, 2004**). We examined the level of activated NF-κB in human breast tumor specimens:

i) by *DNA-binding activity* to the classical response element by EMSA and

ii) by detection of the active complex in the frozen tissue sections by *immunofluorescence assay.*

Tumor Type	Receptor Status	Sample Number	Activated NF-κB (%)
Class I	erbB2-Positive & ER- Negative	7	(86%)
Class II	erbB2-Negative & ER- Negative	9	(33%)
Class III	erbB2-Positive & ER- positive	8	(12%)
Class IV	erbB2-Negative & ER-Positive	7	(14%)

Elevated level of NF-κB is detected predominantly in estrogen receptor-negative human breast cancer specimens, majority being ErbB2 positive . These results corroborated those observed in human and mouse mammary epithelial cell culture systems. Furthermore differential compartmentalization (epithelial vs stromal) of activated NF-kB is detected in 2 different classes of ER-negative tumor specimens (see Fig. 2 Biswas et al. 2004).

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Manuscript submitted to Cancer Research (Singh et al. September, 2005)

The role of NFkB in ER-negative and erbB2-overexpressing human breast tumor cells in culture, was demonstrated by blocking its activation with the NEMO binding domain (NBD) peptide, a specific inhibitor of the inhibitory kappaB (IkB) kinase (IKK). Treatment with NBD reduced cell proliferation, increased apoptosis and the proapoptotic forkhead transcritption factor FOXO3A. NBD used in combination with trastuzumab, a specific inhibitor of the cell surface receptor erbB2, demonstrated synergistic inhibition of cell proliferation at concentrations that singly were ineffective. The low molecular weight IKK inhibitor PS1145 and the proteasome inhibitor PS341 (Bortezomib, Velcade) also blocked cell proliferation and elevated apoptosis and FOXO3A. In combination with trastuzumab, both PS1145 and PS341 synergistically inhibited cell proliferation at low concentrations. IKK-mediated activation of proliferative NFkB and inactivation of pro-apoptotic FOXO3A accompanies imbalanced cell proliferation and death in ER-negative human breast cancer. The reversal of these cancer-associated

processes by the targeted inhibitors provides evidence for the role of activated NFkB in mammary epithelial cell carcinogenesis, particularly in a subclass of ER-negative human breast cancer. These observations reveal a potential potent combinational therapeutic approach for the erbB2-overexpressing ER-negative human breast cancers.

Unpublished results of the ongoing work on this project:

Role of NF-kB signaling in Human Breast Cancer.

To establish the role of activated NF-kB in mammary epithelial cell carcinogenesis we have recently undertaken 2 experimental approaches.

One, by blocking with specific inhibitors of the kinase (IKK) involved in the activation of NF-kB. Our recent results demonstrated reduced cell proliferation, elevated apoptosis and loss of tumorigenic potential (**Slide 2**) following such inhibition of NF-kB activation in ER-negative human breast cancer cells in culture, SKBr3 and MDA-MB-231. These results demonstrate that activated NF-kB is responsible for the transformation phenotypes of these cells and thus qualifies as a potential therapeutic target for subclasses of human breast cancer.

Secondly, we undertook a reverse experimental strategy to establish the role of NF-kB in mammary epithelial cell carcinogenesis. Human breast cancer cells and immortalized normal mammary epithelial cells are transfected with a constitutively activated NF-kB. The transformation phenotypes of these transfected cells are then monitored. Breast cancer cells such as SKBr3 cells do not form tumor in nude mice and form tumors only in the presence of matrigel that is also after a 10-12 weeks of lag period. Such SKBr3 cells stably carrying constituvely activated NF-kB form tumor in nude mice within 4 wks after implantation **(Slide 3)**. These results suggest that constitutive overactivation of NF-kB plays a role in the transformation of these cells aggressively tumorigenic and thus qualifies as a potential therapeutic target.

Project 6. Development of Nuclear Receptors as Targets for Prevention

Antiestrogens have been shown to reduce the risk of invasive estrogen receptor (ER)-positive breast cancer in high-risk women without pre-existing cancer. However, prevention strategies for ER-negative breast cancers, which constitute over 30% of breast cancers, are needed. Increases in various cell cycle regulatory proteins, including the oncogene product cyclin D1, have been demonstrated in all breast cancers, including ER-negative tumors. Transgenic mice with overexpression of cyclin D1 have been found to have an increased incidence of mammary adenocarcinomas. Activation of the peroxisome proliferator-activated receptor g (PPARg), a nuclear receptor present in breast and other tissues, leads to inhibition of cyclin D1. Thiazolidinediones, a class of drugs that includes rosiglitazone, are selective PPARg agonists that have been shown in *in vitro* studies to reduce cyclin D1 protein expression and subsequently result in cell cycle arrest in G1 phase and decreased cell proliferation. We want to study the effects of rosiglitazone on cyclin D1 expression in women at high risk for breast cancer by measuring cyclin D1 expression in cells obtained by ductal lavage from high-risk women before and after treatment with rosiglitazone for one month.

We obtained 18 preliminary ductal lavage specimens from 13/21 women from at least one breast. The specimens were divided into two portions for cytological analysis and RNA isolation. Two of the specimens were classified as atypical. We determined the RNA yield and levels of expression of a series of relevant genes including PPARγ, cyclin D1, and cell-type specific markers in the lavage samples using quantitative real-time PCR. In addition, we began to look at the variation of marker gene expression in untreated women in repeat samples over time. Most of the ductal lavage specimens yielded an average of 65ng RNA, which was adequate for the measurement of PPARg and cyclin D1, as well as a number of other molecular markers. By using cell-type specific markers we were able to estimate the

cellular composition of the lavage samples, which consist of luminal epithelial cells, basal/myoepithelial cells and leukocytes. We used a clustering program to show the relative expression of each molecular marker in the ductal lavage specimens and how the specimens compare to each other. These preliminary results suggest that a biomarker trial should be feasible analyzing ductal lavage specimens for alterations in PPARgand cyclin D1 expression from 20 women before and after treatment with rosiglitazone. The results of this study will further the assessment of thiazolidinediones as preventive agents in breast cancer.

Core 1. Signal Transduction Rapid Assessment Core

The objectives of this core remain the establishment of a robust technology that allows one to query the activation status of signal transduction pathways relevant to ER-negative breast cancer. We have been exploring the use of the Luminex technology. We have continued to develop and obtain phospho-specific antibodies to the relevant proteins and have been piloting their adaptation to the Luminex platform. In addition we have begun to explore back-up technologies should this platform not be able to perform as expected.

Core 2. Communications Core

This core supports the entire project in several areas including Communications, Administration, Informatics and Biostatistics. In terms of Communications, we have continued to develop and use the COE web site (http://research.dfci.harvard.edu/coe). We have been holding monthly work-in-progress meetings in which each project has had the opportunity to present their progress and for problems to be discussed. In addition the Informatics and Biostatistics components have been actively involved in several of the projects. Thus far, statisticians in the core have collaborated with investigators from projects 3,4, and 6 (and we note that projects 1 and 2 have their own statisticians among their investigators, although project 2 in the future will also involve work by some statisticians in the communications core). On project 3, an analysis of ErbB2 cDNA arrays which examined the effect of TGFbeta, AP1510, and the ratio of these two growth factors was done. Locally written software (Bioconductor) was used to normalize the array values and then a linear model involving time and these 3 terms was done for each of the 895 spots that remained in the analysis after filtering. On project 4, the relationship of gene expression to clinical and pathologic characteristics and the association between the expression of different candidate genes were evaluated on eight separate microarray data sets using logistic regression (based on dichotomizing expression into positive or negative). This analysis was included in the 2003 paper in Molecular Cancer Research by Porter, et.al. An analysis of Hid5 immunostaining and its relationship to clinical variables, HER2 expression, and IGF-1R pattern, location, and intensity of staining was also done for project 4. In addition, a statistical design was developed for a matched triplicate study of whether the PTEN/AKT pathway differs between tumors from BRCA1 mutation carriers and tumors from other ER negative, Her2 negative breast cancer patients. On project 6, two experiments on mice that had either wild type PPARgamma or were heterologous for PPARgamma involved different schedules for sacrifice and were analyzed to see if the earlier sacrifice schedule could be used (that is, if it didn't make any difference in the estimated genetic association with time to tumor or time to multiple tumors within an animal). The conclusion was that the earlier sacrifice schedule could be used. In addition, for this project several sample size calculations have been done for planned ductal lavage clinical protocols.